Practitioner's Docket No. 142/003/PCT *PATENT*

Preliminary Classification:
Proposed Class:
Subclass:

**TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE (DO/US)--
ENTRY INTO THE U.S. NATIONAL STAGE UNDER CHAPTER I**

25109 RP

142/003/PCT/US99/23715	26 October 1999 (26.10.99)	26 October 1998 (26.10.98)
International Application Number	**International Filing Date**	**International Earliest Priority Date**

TITLE OF INVENTION: Detection of Human Papilloma Virus in Papanicolau (Pap) Smears

APPLICANT(S) FOR DO/US: Light, Elizabeth S.; Nuovo, Gerard

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: DO/US

1. Applicant herewith submits to the United States Designated Office (DO/US) the following items under 35 U.S.C. Section 371:

This express request to immediately begin national examination procedures (35 U.S.C. Section 371(f)). The U.S. National Fee (U.S.C. Section 371(c)(1)) and other fees (37 C.F.R. Section 1.492), as indicated below:

CERTIFICATION UNDER 37 C.F.R. SECTION 1.10*
*(Express Mail label number is **mandatory.**)*
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date **June 26, 2000**, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **EL262636000US**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Tandala R. Nobles
(type or print name of person mailing paper)
Tandala R Nobles
Signature of person mailing paper

2. Fees

CLAIMS FEE*	(1) FOR	(2) NUMBER FILED		(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS	22	-20 =	2	x $18.00 =	$36.00
	INDEPENDENT CLAIMS	1	- 3 =	0	x $78.00 =	$0.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $260					$0.00
BASIC FEE	The international search fee, as set forth in Section 1.445(a)(2) to be paid to the US PTO acting as an international Searching Authority: has been paid (37 CFR 1.492(a)(2)) $690.00					$690.00
	Total of above Calculations					= $726.00
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed (note 37 CFR 1.9, 1.27, 1.28).					- $363.00
	Subtotal					$363.00
	Total National Fee					$363.00
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item below). See attached "ASSIGNMENT COVER SHEET".					$0.00
TOTAL	Total Fees enclosed					$363.00

Please charge Account No. 50-0861 in the amount of $363.00.
A duplicate copy of this sheet is enclosed.

3. A copy of the International application as filed (35 U.S.C. Section 371(c)(2)) is not required, as the application was filed with the United States Receiving Office.

4. A translation of the International application into the English language (35 U.S.C. Section 371(c)(2)) is not required as the application was filed in English.

5. An oath or declaration, including power of attorney, of the inventor (35 U.S.C. Section 371(c)(4)) complying with 35 U.S.C. Section 115 is submitted herewith, and such oath or declaration is attached to application.

II. Other document(s) or information included:

6. An international Search Report or Declaration under PCT Article 17(2)(a) is not required, as the application was searched by the United States International Searching Authority.

7. An Information Disclosure Statement under 37 C.F.R. Sections 1.97 and 1.98 will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).

Date: June 26, 2000

Signature of Practitioner

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Practitioner's Docket No. 142/003/PCT *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Light, Elizabeth S.; Nuovo, Gerard J.

Application No.: PCT/US99/~~23815~~ 25109

Filed: 26-OCT-1999

For: **Detection of Human Papilloma Virus in Papanicolau (Pap) Smears**

STATEMENT
CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(c-f) and 1.27(b-d))

Assistant Commissioner for Patents
Washington, D.C. 20231

With respect to the invention described in application no. PCT/US99/23815, filed October 26, 1999.

I. IDENTIFICATION AND RIGHTS AS A SMALL ENTITY

I hereby state that I am an official of the small business concern empowered to act on behalf of the concern identified below:

Ventana Medical Systems, Inc.
3865 N. Business Center Drive
Tucson, AZ 85705

and that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

II. OWNERSHIP OF INVENTION

I hereby state that rights under contract or law remain with and/or have been conveyed to the above identified organization

EXCEPT, that if the rights held are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held (1) by any person who could not

be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, (2) any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or (3) a nonprofit organization under 37 CFR 1.9(e).

III. ACKNOWLEDGEMENT OF DUTY TO NOTIFY PTO OF STATUS CHANGE

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

IV. DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

V. SIGNATURE

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SIGNATURE  DATE 6/26/00

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WO 00/24760 PCT/US99/25109

DETECTION OF HUMAN PAPILLOMA VIRUS IN PAPANICOLAOU (Pap) SMEARS

FIELD OF THE INVENTION

The present invention relates to methods and reagents to detect viral DNA in atypical PAP smears indicative of predisposition for cancer.

BACKGROUND OF THE INVENTION

Human Papilloma Virus (HPV) is the most common sexually transmitted viral disease and is manifested in over 1 million people per year in the United States. There are at least 90 distinct types of HPV of which over 25 are found in the lower genital tract. Of the HPV types found in genital lesions, some are implicated in cervical precancers and cancers.

These types are ranked according to a high, intermediate or low risk or tendency to progress to cancer.
Common high risk types include: 16, 18, 39, 45, 56 etc., intermediate risk types include: 31, 33, 35, 51, 52, 58 etc., and low risk types include: 6, 11, 42, 43, 44 etc.

Human Papilloma Virus (HPV) is a family of DNA viruses where the genome is about 7900 base pairs having seven open reading frames expressed early in infection and two expressed late in infection. Protein E6 and E7 are linked to inducing transformation of benign cells in vitro and in vivo. While the exact mechanism is yet to be proven, one of the proteins from high-risk strains appears to inactivate the human tumor suppressor gene p53.

Currently, Pap smears are performed yearly on women to check for a presence of atypical or cancerous cells. Roughly 90% of all smears are normal, 3% are unequivocally dysplastic including LSIL and 7% are squamous atypias (ASCUS). Patients with a diagnosis of dysplasia are brought back to their

doctor's office for biopsy and further procedures. Normal results are untreated. The ASCUS and LSIL diagnoses present the doctor and patient with multiple choices for treatment. If these patients could be tested for high risk type HPV presence it would provide further information on the best treatment route. The presence of a low risk HPV type could indicate no further action except perhaps more frequent pap smears. A high risk HPV type presence could indicate a more aggressive approach: either a cone biopsy, LEEP (loop electrode excision procedure), or ablative therapy (laser surgery or cryotherapy).

An alternative to standard Pap smears is the Cytyc Corporation's ThinPrep test. A standard Pap smear involves sampling the uterine cervix with a spatula or cytobrush and smearing the cells directly onto a glass slide. The ThinPrep test involves suspending the sample in a buffered fixative solution which provides better sample recover, fewer artifacts, multiple slides from one sample, less cell crowding, overlap and better cellular morphology.

In a ThinPrep sample, the sampling device is rinsed into a buffered preservative solution and this solution is used to make slides. By suspending the sample in preservative solution and using the ThinPrep machine to make the slides, the resulting slide is a thin-layer preparation that is cleaner of blood, inflammatory cells, mucus, and other obscuring artifacts than a standard Pap smear. The FDA has found the ThinPrep test to be preferable to the standard Pap smear for detection of LSIL and more severe lesions. Another advantage of the ThinPrep test is that the entire sample is not used to make a single slide. The preserved cells can be used to make more slides on which HPV testing could be performed.

The standard current methods for detecting cervical cancer is the PAP smear or Cytec's ThinPrep. Both detect atypical or cancerous cells, not HPV infection. PAP smears are classified as:

- normal
- ASCUS (atypical cells of uncertain significance)
- LSIL (low-grade squamous intraepithelial lesion)
- HSIL (high-grade squamous intraepithelial lesion)

Currently about 4.4 million PAP smears are classified as ASCUS per year. 2.5 million PAP smears are classified as LSIL per year. This indicates that 6.9 million or 7-8% of all PAP smears per year are ambiguous in their results. Knowledge of which HPV type present would aid the patient and clinician in determining risk level and treatment options.

Patients with an ambiguous cytology may still have preinvasive or microinvasive cancer and HPV DNA typing may aid in differentiating patients. Studies have shown that ambiguous cytology and high risk HPV infection with types 16, 18 and 31 are more likely to have high grade SIL or microinvasive histopathology on biopsy. Studies have also suggested that acute infection with HPV types 16 and 18 confer an 11 to 16.9 fold risk of rapid development of CIN.

Accordingly, a need exists to differentiate the borderline Pap smears and ThinPrep slides based on the HPV type present, if any. Other attempts at preparing oligonucleotide probes have been reported. See U.S. Patents 5,679,509, 5,057,411, Cole et al, Journal of Molecular Biology 193: 599-608 (1987), 5,554,538, 5,484,699, 5,501,947, 4,849,332, 5,411,847, 4,908,306, EP 0,477,972 B1, 5,527,898, 4,983,728, 4,849,334, 4,849,311 and 4,820,530. Applicants are also aware of U.S. Patent 5,538,871. However, each oligonucleotide probe appears to be specific to only one type, thus, many would be needed to form a complete set to detect all high risk HPV types.

All patents and references cited herein are explicitly incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a method for detecting HPV infection by high risk or low risk HPV types in individual cells by in situ hybridization.

In a related aspect, the invention relates to a reagent comprising a plurality of full length genomic probes to certain high risk HPV types or a second reagent comprising a plurality of full length genomic probes to certain low risk HPV types.

In a related aspect, the invention relates to exploiting the cross-reactivity of the probes according to the present invention to determine whether an HPV infected cell has any HPV type which is associated with malignancy not only those HPV types completely complementary to the probes.

In another aspect, the invention relates to methods correlating HPV type detection with the conventional cytology characterization as a second factor indicating whether the cells are normal or abnormal.

The present invention achieves these results by using a set of six essentially full length genomic probes in certain proportions which under low stringency cross react with all 13 currently known high risk HPV types and none of the low risk HPV types. The probes are labeled so that the labeling system may be visualized using conventional light microscopy which can also separately or simultaneously determine cell morphology as a measure of potential neoplasia. Alternatively, one may use a fluorescent labeling system and fluorescence microscopy to detect the results.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The protocol of the present invention was developed on Pap smears and ThinPrep samples. This system may also be applied for tissue sections. The samples may be previously stained or embedded in paraffin provided that they are

destained or deparaffinized before use. Deparaffination may be performed by heat and detergent or solvent (e.g. xylene).

The probes being used are essentially full length genomic HPV probes specific for HPV types 16, 18, 31, 33, 35 and 51. The full length probes of the present invention have essentially the same sequence as given in GenBank Accession Numbers: K02718 - type 16; X05015 - type 18; J04353 - type 31; M62877 - type 51; M12732 and A12360 - type 33; M74117 - type 35. While some sequence variations and shortening of the probe length are permitted, these are still considered full length and are not similar to oligonucleotide probes as used in the prior art.

These probes range from roughly 6000 to 8000 base pairs each and are mixed together forming a reagent which is used with a hybridization cocktail using low stringency hybridization solution and conditions and lower stringency post wash solution and conditions. This permits one to detect the 13 known high risk types. The probe cocktail was tested on samples of known HPV types to be sure of detecting all high risk types with no cross hybridization to the low risk types. The results are provided in the attached manuscript and show a very good signal and low to no background.

The probes are labeled with digoxigenin or fluorescein by nick translation; however, a very wide variety of other labeling techniques may be used. Examples include, incorporation during PCR and random priming. The probes need not be labeled at all if one uses an anti-duplex or anti thymidine dimers antibody.

The in situ hybridization and detection according to the present invention has several advantages over other methods. First, since the present invention is an in situ hybridization, the cytology of the individual cell can be correlated with HPV positive or negative results. Second, since amplification of DNA by PCR, hybrid capture or other amplification techniques is not needed, the system is less subject to contamination or background yielding false positive or negative results. Third, the processed slides can be

stored for years for later reference or confirmation. Fourth, since the reaction is performed on currently used patient sampling methods, no changes need to be instituted at the clinician level. A corollary to this advantage is that the reaction can be performed on destained Pap smears, so the patient does not need to return to the clinician's office for a second sampling in the event of an ambiguous Pap smear cytology. Fifth, the manipulation of the current probe set allows detection of currently classified and unclassified high risk HPV types. Alternatively, if a ThinPrep tube sampling was performed, the same cell suspension can be used to create a slide for the in-situ reaction. This allows the extra advantage that the system does not need reformulation to include newly identified HPV types. Sixth, one can differentiate between a few highly positive cells and many weakly positive ones to differentiate between a clinically important or subclinical infection, though ASCUS may be clinically important for reasons other than HPV.

The sample slide is processed through a series of steps to allow target DNA on the sample to hybridize to the probe without losing the morphology of the cell. See the method of PCT WO94/09022 and U.S. Patent Application 08/272,315, filed July 22, 1994. This includes a Proteinase K digestion and subsequent wash and dehydration steps. The digoxigenin labeled probe and target DNA are codenatured for 5 minutes at 95°C on a hot plate and then hybridization is allowed to proceed for three hours to overnight at 37°C. Following a post-hybridization wash, the slide is incubated with horseradish peroxidase (HRP) conjugated anti-digoxigenin antibody for 30 minutes at 37°C. The detection scheme utilizes the TrueBlue peroxidase substrate (KP Labs, Gaithersburg, MD). Alternatively, anti-digoxigenin antibody labeled with alkaline phosphatase with NBT/BCIP as chromogen was also used.

A thirty minute incubation of the TrueBlue substrate with the HRP detected probe produces a dark blue precipitate at the hybridization site. The cells were then counterstained with

eosin, a pink cytoplasmic stain which contrasts well with the TrueBlue reaction product. The results are visualized using brightfield microscopy and can either be imaged or photographed. The slides can be stored indefinitely without losing signal. Elapsed time to set up the slide for hybridization is roughly 35 minutes and the post-hybridization elapsed time before the slide can be visualized is about 65 minutes.

Alternatively, one may use an automated protease digestion and subsequent wash steps. The probe and target DNA are codenatured for 5 minutes at 90°C on a hot plate and then hybridization is allowed to proceed for one hour to overnight at 37°C. Following a post-hybridization wash, the slide is incubated with alkaline phosphatase conjugated anti-digoxigenin antibody for 30 minutes at 37°C. The detection scheme utilizes an alkaline phosphatase substrate NBT/BCIP (Ventana Blue, Gaithersburg, MD). Alternatively, anti-fluorescein antibody labeled or subsequently conjugated with horseradish peroxidase with TMB as chromogen or alkaline phosphatase with NBT/BCIP as chromogen may also be used.

A thirty minute incubation of the Ventana Blue substrate with the alkaline phosphatase detected probe produces a blue/black precipitate at the hybridization site. The cells were then counterstained with eosin, a pink cytoplasmic stain which contrasts well with the NBT/BCIP reaction product. The results are visualized using brightfield microscopy and can either be imaged or photographed. The slides can be stored indefinitely without losing signal. Elapsed time to set up the slide for hybridization is roughly 25 minutes and the post-hybridization elapsed time before the slide can be visualized is about 90 minutes.

Brightfield detection is preferable to fluorescence because more laboratories have the necessary equipment, personnel are more familiar with the equipment and observation of cell morphology under brightfield detection, the analysis is easily automated and slides are readily preserved without

signal loss. Other labeling systems, e.g. fluorescence, etc., is also an acceptable alternative.

The advantages of brightfield detection over fluorescent detection are important in this application. These slides could be preserved without signal loss due to fading, analysis could be more easily automated, and the morphology of the infected cells could be seen by the cytopathologists and cytotechnicians performing the test.

With a positive high risk HPV in situ result, the clinician may recommend another test every six months and may suggest a minor surgical treatment such as LEEP but is most likely to recommend colposcopy, probably with a biopsy.

The probes and reagents of the present invention are preferably packaged in kit form containing the probes mixed together as a single reagent in a container. Other reagents for sample treatment, hybridization and wash solutions, label detection systems and developing reagents for the label detection systems may also be incorporated in one or more other containers.

The following examples are included for purposes of illustrating certain aspects of the invention and should not be construed as limiting.

EXAMPLE 1: PROBE SYNTHESIS AND ABILITIES

Six separate plasmids were prepared, one for each HPV type, with one plasmid containing the whole genome of a HPV type and the six types being types 16, 18, 31, 33, 35, and 51. The HPV was cloned into a plasmid by standard molecular biology techniques and are within the skill of the art. These plasmids were labeled by nick translation with digoxigenin dCTP. The labeled plasmids were then mixed together to form a single reagent. Incorporation of the digoxigenin nucleotide into the labeled DNA was verified by a dot-blot procedure.

The ability of each probe to cross-react with other high risk HPV types known to be associated with malignancy and not

to cross react with low risk HPV types not associated with malignancy was tested. The data is given below in TABLE 1 where the probes used are listed as rows and the Pap smears ha e the HPV strain listed in the columns.

TABLE 1

	6/11	16	18	31	33	35	39	41	42	43	44	45	51	52	56	58	59	68	70
16	2	X	0	1	½	½	0	0	1	½	1	0	½	0	0	1	0	0	0
18	0	0	X	0	0	0	1	½	0	0	0	2	0	0	½	0	1	1	1
31	1	1	0	X	1	2	0	0	½	0	0	0	1	0	1	1	0	0	0
33	0	½	0	1	X	½	0	0	0	0	0	½	0	1	0	2	0	0	0
35	½	½	0	1	½	X	0	0	0	0	0	0	0	2	½	½	0	0	0
51	0	½	0	1	0	0	0	0	0	0	0	0	X	0	0	0	0	0	0

0: No Detection ½: Barely Detected 1: Light Blue
2: Moderate Blue 3(X): Dark Blue

The probe to type 70 was not needed since all high risk HPV types are covered by other probes. Thus, it was not actually used in the reagent of the present invention.

Since some undesired cross reactivity was noted with probes to HPV type 16 and 31, the concentration of these two was lowered in the probe reagent to compensate. The percentages of each genomic probe in the DNA cocktail are given in TABLE 2.

TABLE 2

HPV 16	HPV 18	HPV 31	HPV 33	HPV 35	HPV 51
8.3%	20.8%	8.3%	20.8%	20.8%	20.8%

The resulting data was compared against 57 patient samples and resulted in the data of TABLE 3 where the cross reacting to the low risk types was essentially nullified. Comparison was made to another commercial HPV probe product.

TABLE 3

HPV type	Digene Probe	Present Probe Cocktail
2	3+	0
6/11	1+	0
6/11	1+	0
6/11	2+	0
6/11	3+	0
6/11	3+	1+
6/11	3+	0
6/11	3+	0
6/11	3+	0
6/11	3+	0
6/11	1+	0
6/11	1+	0
42	2+	0
43	2+	0
44	2+	0
16	1+	1+
16	1+	1+
16	2+	2+
16	Weak	1+
16	3+	3+
16	3+	3+
18	3+	3+
31	2+	2+
31	3+	3+
31	3+	3+
33	3+	3+

33	2+	2+
25	3+	3+
35	1+	-
51	3+	3+
30	3+	3+
30	3+	3+
30	3	3+
39	2	1+
39	2	2+
39	1	Weak
39	3	3+
39	3	3+
39	1	1+
45	3	3+
45	3	3+
52	3	3+
52	3	3+
52	2	2+
56	3	3+
56	1	1+
56	3	2+
56	3	1+
58	2	1+
59	2+	2+
68	1+	1+
70	3+	3+
70	2+	2+
70	1+	1+
still novel	2+	2+
still novel	1+	1+
still novel	1+	1+

As shown above, the present probe cocktail was shown to not give false positives with low risk HPV types unlike the probes of a competitor. Thus, the present invention will give a lower false positive reaction.

Furthermore, when both probe sets were applied to normal Pap smears containing HPV, the Digene probes indicated a positive reaction whereas the coctail of the present invention did not yield a strong positive reaction. This is an important check as occasionally, Pap smears are misread. It is generally thought that ten percent or more of the population is infected with HPV but this only poses a risk in the presence of displasia or other morphological abnormality. Again, the present invention yields fewer false positives.

EXAMPLE 2: SAMPLE PREPARATION AND PRETREATMENT

Uterine cervix cells were sampled and smeared to form a conventional Pap smear or suspended in PreservCyt (Cytyc Corporation), a buffered fixative and preservative solution. The ThinPrep 2000 (Cytyc Corporation) was used in make two ThinPrep slides for each patient. One slide was stained for conventional cytology similar to that of conventional PAP smears and the other slide was prepared as below.

A variety of protease treatments were attempted. The effect of varying the concentrations of proteinase K and pepsin on the intensity of the in situ hybridization signal with an alu probe on archival, destained Pap smears results are given in TABLE 4. Archived Pap smears were deparaffinized by submersion in xylene and washed in ethanol. Destaining was accomplished by washing the slides for 20 minutes at room temperature in 70% ethanol and 0.1 N HCl. The slides were then washed in tap water for 10 minutes, rinsed in 100% ethanol for 5 minutes, and air dried. All samples were digested for 20 minutes at 37°C. The post hybridization wash was done at high stringency (60°C, 0.2X SSC and 2% BSA. The following procedure was used on patient samples.

TABLE 4

Protease Concentration	0	1 (μg/ml)	10	25	50	100	200	2,000
Proteinase K								
in water	+/-	1+	3+	3+	3+	2+	1+	OD
in 1XSSC	+/-	3+	1+	OD	OD	OD	OD	OD
Pepsin								
in water	+/-	1+	2+	2+	3+	3+	2+	OD
in 0.1 N HCl	+/-	0	1+	1+	2+	2+	3+	OD

Signal intensity was scored by the percentage of positive cells (,25%=1+, 25-50%=2+, >50%=3+). OD means overdigested, in which cell morphology is poor and no signal is evident.

The Pap smear and the ThinPrep slide were incubated for 20 minutes at 37°C in a solution of 10 micrograms per milliliter of Proteinase K in 2X SSC. Following the incubation, the slide was washed for 2 minutes at room temperature in 2X SSC, dehydrated in a series of 70%, 80%, and 95% room temperature ethanol solutions for 1 minute each and air dried.

EXAMPLE 3: PROBE AND TARGET HYBRIDIZATION

A probe solution was made consisting of 0.5 nanograms per milliliter of HPV types 18, 33, 35, and 51 and 0.2 nanograms per milliliter of HPV types 16 and 31 in Hybrisol IX (Ventana) using the probes prepared above. Ten microliters of this probe solution was pipetted onto the sample slide and the specimen was covered with a 22 mm round coverslip and optionally sealed with rubber cement. The slide was placed on a prewarmed 95°C hot plate for 5 minutes to denature the probe and target DNA and then transferred to a humidified chamber and placed in a 37°C incubator. The slide was incubated at 37°C in the humidified chamber for 2 to 16 hours to hybridize. After the 37°C incubation for hybridization, the rubber cement

and coverslip are removed. The slide is washed and the signal detected.

Both high stringency post hybridization wash conditions (0.2X SSC, 2% BSA, 60°C, 10 minutes) and low stringency post hybridization wash conditions (2X SSC, 2% BSA, 45°C, 10 minutes) were used on a number of patient samples for whom the HPV type was determined. The data is given in TABLE 5 where detection of different HPV types in cervical biopsies by the high risk HPV consensus probe as a function of stringency of the post hybridization wash.

TABLE 5

	HPV DNA POSITIVE	
HPV type	Low Stringency	High Stringency
Common HPV types		
6/11	0/11	0/11
16/18	11/11	11/11
31/33/35	7/7	7/7
51	4/4	4/4
Rare HPV types		
42/43/44	0/3	0/3
30	3/3	1/3
39	7/7	2/7
45	3/3	3/3
52	3/3	1/3
56	4/4	1/4
58/59/68	3/3	1/3
70	3/3	3/3

Low stringency is a post hybridization wash of 10 minutes in 2X SSC with 2% BSA at 45°C.
High stringency is a post hybridization wash of 10 minutes in 0.2X SSC with 2% BSA at 60°C.

These common HPV types listed comprise about 75% of those types found in lower grade cervical SILs (4-6). Each of the rare HPV types is found in from 1-3% of low grade cervical SILs (4-6). Note that all of the patient samples with high

risk HPV types associated with malignancy were detected under low stringency and none of the low risk HPV types not associated with malignancy were detected under low stringency.

EXAMPLE 4: IMMUNOCHEMICAL DETECTION

The sample on the slide was then incubated with horseradish peroxidase (HRP) labeled anti-digoxigenin antibody (Boehrigher Mannheim GMBH). The slide was washed three times for two minutes each in 1X PBD to remove any unbound or loosely bound antibody. The slide was removed from 1X PBD and allowed to drain briefly. Two hundred microliters of TrueBlue Peroxidase Substrate™ (KPL) was added to the slide and the reaction proceeds at room temperature for three minutes. The slide is rinsed in distilled water and allowed to air dry.

The slide was dipped in a 1/4X solution of Eosin in ethanol to counterstain. The slide was rinsed three times in distilled water and allowed to air dry. To mount, the slide was dipped in xylenes and a drop of Permount (Fisher) is added. The slide is then covered with a 22 mm round glass coverslip.

Cells with high risk HPV integrated in the cell demonstrate a blue precipitate in the nuclei with minimal slide background. The cytoplasm is counterstained pink for contrast. Cellular morphology confirms that high risk HPV types were was present in a cell which would be classified as abnormal and normal cells lack any positive signal.

Alternatively, alkaline phosphatase labeled anti-digoxigenin antibody may be used along with NBT/BCIP detection reagents as is used in the attached manuscript.

EXAMPLE 5: COMPARISON OF PAP SMEAR RESULTS TO HPV TYPE TESTING

A sizable number of normal, ASCUS and SIL PAP smears were tested for HPV type status. Those detected by the probe reagent of the present invention are indicated as positive cases. The detection of HPV DNA using the high risk consensus

probe at low stringency conditions as a function of the cytologic diagnosis and, for cases of ASCUS, clinical follow up data is given in TABLE 6.

TABLE 6

Pap Smear Result	HPV Positive
Cases	
Normal	1/19 (5%)
ASCUS (total)	16/40 (40%)
SIL	18/23 (78%)
ASCUS (biopsy of SIL within 6 months)	14/21 (67%)
ASCUS (biopsy negative for SIL within 6 months)	2/19 (10%)

The one normal positive was rescreened by two cytotechnologists who did not know the HPV result and was classified by each as ASCUS. The ASCUS subgrouping was statistically significant difference at p=0.05 using the nonparametric two-tailed Mann-Whitney test as per InStat, Version 2.0 (GraphPad Software, San Diego, CA).

EXAMPLE 6: TESTING CELL LINES

The probes above were tested against three known cell lines to confirm their ability to detect HPV with respect to the copy number of viruses in each cell line obtained from ATCC. The data is given in TABLE 7.

TABLE 7

Cell Line	HPV type	Copy number	Detected
SiHa	16	1	✔
HeLa	18	20	✔✔
CaSki	16	600	✔✔

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but

merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

What is claimed is:

1. A reagent for detecting human papilloma virus DNA in a cell sample which indicates the patient providing the cell sample is at risk for cancer comprising;

a plurality of DNA probes capable of specifically hybridizing to high-risk HPV DNA but not low-risk HPV DNA.

2. The reagent of claim 1 wherein the probes hybridize to HPV types 16, 18, 31, 33, 35 and 51 but not 6, 11, 41, 42, 43 and 44.

3. The reagent of claim 2 wherein the probes also hybridize to HPV types 39, 45, 52, 56, 58, 59, 68 and 70.

4. The reagent of claim 1 wherein the cell sample is cervical cells taken from a patient.

5. The reagent of claim 1 wherein the DNA probes are full length HPV probes.

6. The reagent of claim 1 consisting essentially of DNA probes to HPV types 16, 18, 31, 33, 35 and 51.

7. The reagent of claim 6 where the each DNA probe is in the following amounts: HPV 16 - 8.3%, HPV 18 - 20.8%, HPV 31 - 8.3%, HPV 33 - 20.8%, HPV 35 - 20.8%, HPV 51 - 20.8%

8. A method for detecting human papilloma virus DNA in a cell sample which indicate the patient providing the cell sample is at risk for cancer comprising;

adding the reagent of claim 1 under hybridization conditions, and

detecting the presence or absence of hybridization inside cells in the cell sample.

9. The method of claim 8, wherein the reagent probes hybridize to HPV types 16, 18, 31, 33, 35 and 51 but not 6, 11, 41, 42, 43 and 44 in a cervical cell sample.

10. The method of claim 8 wherein the reagent probes also hybridize to HPV types 39, 45, 52, 56, 58, 59, 68 and 70 and low stringency hybridization conditions are used.

11. The method of claim 8 further comprising pretreating the cell sample with a protease.

12. The method of claim 8 further comprising destaining and/or deparaffining the cell sample.

13. The method of claim 8 wherein the reagent contains full length HPV probes.

14. The method of claim 8 wherein the reagent consisting essentially of DNA probes to HPV types 16, 18, 31, 33, 35 and 51.

15. The method of claim 14 where the reagent contains DNA probes in the following amounts: HPV 16 - 8.3%, HPV 18 - 20.8%, HPV 31 - 8.3%, HPV 33 - 20.8%, HPV 35 - 20.8%, HPV 51 - 20.8%

16. The method of claim 15 wherein the cell sample contains abnormal cervical cells.

17. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 1.

18. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 2.

19. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 3.

20. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 5.

21. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 6.

22. A kit for detecting high and intermediate risk human papilloma virus DNA in a sample comprising a container containing the reagent of claim 7.

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(54) Title: DETECTION OF HUMAN PAPILLOMA VIRUS IN PAPANICOLAOU (Pap) SMEARS

(57) Abstract

Methods and reagents for detecting higher risk human papilloma virus DNA types in cells on a Pap smear which indicates the patient is at higher risk for cancer are described. The method uses full length DNA probes to HPV types (16, 18, 31, 33, 35, and 51) is a particular proportion to hybridize to and detect the viral DNA in-situ. The method differentiates high risk from low risk human papilloma virus DNA in cells which indicates the patient's risk for cancer. The in-situ hydridization is detected by brightfield microscopy.

Docket No.: 112163.128 **PATENT/OFFICIAL**

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter claimed and for which a patent is sought on the invention entitled:

DETECTION OF HUMAN PAPILLOMA VIRUS IN PAPANICOLAOU (Pap) SMEARS,

the specification of which

[ ] is attached hereto OR
[X] was filed on 26 June 2000 as Application Serial No. 09/582,492.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			**Priority Claimed**	
Number	**Country**	**Day/Month/Year filed**	**Yes**	**No**

I hereby claim the benefit under 35 USC 119(e) of any United States provisional application(s) listed below.

Prior Provisional Application(s):

Application Number	**Filing Date**
60/105,657	26 October 1998

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. Non-Provisional Application(s):

Serial No.	**Filing Date**
PCT/US99/25109	26 October 1999

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby grant the firm of HALE AND DORR LLP the power to insert on this Declaration any further identification, including the application number and filing date, which may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for recordation of this document.

I hereby appoint Huw R. Jones, Registration No. 33,916, a registered practitioner listed at customer number 23874, and all other practitioners associated with customer number 23874, having the following address:

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